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Identification of environmental stress biomarkers in seedlings of European beech (*Fagus sylvatica* L.) and Scots pine (*Pinus sylvestris* L.)

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Identification of environmental stress biomarkers in seedlings of the European beech (*Fagus sylvatica* L.) and the Scots pine (*Pinus sylvestris* L.)

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Abstract

Climate development models predict alterations that will critically influence plant metabolism in Southern and Central Europe. Although the molecular players involved in the response to climatic stress factors have been well described in crops, little information is available for forest tree species. Consequently, the identification of molecular biomarkers suitable for evaluating the actual impact of different environmental stress conditions on forest plants would be of great importance for monitoring purposes and forest management. In this study we evaluated a biochemical methodology for the assessment of temperature stress in European beech (*Fagus sylvatica* L.) and Scots pine (*Pinus sylvestris* L.) seedlings by analyzing a set of metabolites and enzymes involved in free radical scavenging and cell wall synthesis. The results indicate that the combined analysis of the specific activities and isoform profile of peroxidases, superoxide dismutases, and glutathione peroxidases coupled with the amount variation of phenolic compounds enabled to discriminate between stressed and control seedlings. This approach represents a promising platform for the assessment of temperature stress in forest trees and could also enhance selection and breeding practices allowing for plants more tolerant/resistant to abiotic stress.

Key words: Abiotic stress, Enzyme activity, Forestry, Peroxidase, Temperature stress

1. Introduction

Recent studies of global climate change suggest that there will be more intense, more frequent, and longer lasting heat in upcoming climate. For example, coupled models of climate and terrestrial biosphere functions predict a continuous increase in average global temperatures of between 1.5 and 4°C/100 years, with CO₂ concentrations rising to between 800 and 1000 ppm. (Gunderson et al., 2000; Friedlingstein, 2006; Heyder et al. 2011). Environmental stress factors such as drought and over-irradiation due to natural conditions as well as to agronomic practices such as selective pruning or forest thinning that influence albedo and temperature of the uncovered soils can be expected to become increasingly critical for forest trees growing in central Europe. Changes in temperature are among the most important factors that affect early plant development and their health. Plants have evolved mechanisms to monitor their environment and to respond at cellular, physiological, and morphological level to optimize growth and reproductive success within their lifespan (Taiz and Zeiger. 2010). Measurements of variations in enzymatic activity, substrate specificity, and tissue localization of key enzymes represent valuable approaches to assess the intensity of applied stress. . Most of the literature concerning stress factors in plants describes data collected using model plants, crops and, rarely, fruit and industrial trees such as poplar (Bray. 2004, Cohen et.al. 2010, Fortunati et.al. 2008, Qureshi et.al. 2007). Only recently the quantitative variation of proteins in European beech grown under ozone stress has been described (Kerner et.al. 2014). The available surveys indicate that three main groups of proteins might be particularly predictive, namely enzymes involved in stress and defense metabolisms, such as PR-2 and PR-10 proteins, as well as storage proteins. Specifically, the plant antioxidant response seems to be rapidly activated by heat (Ferreira et.al. 2006) and cold (Dai et.al. 2009) conditions. In adult beech trees (Haberer et.al. 2007) and in young beech plants (Herbinger et.al. 2007) antioxidative compound variations were studied in a free-air controlled ozone exposure system. However, the identification of reliable protein biomarkers suitable for evaluating quantitatively the actual impact of different environmental stress conditions in forest plants is still to be achieved. Glutathione and the enzymes involved in its turnover have been considered as potential stress

106 biochemical makers of forest tree species because of their role in ozone detoxification (Dizengrem el et.al.
 107 2013) but no experimental evidence has been brought to support this diagnostic approach. Nevertheless,
 108 DNA expression and proteomic data (Gonzalez-Martinez et.al. 2007, Quareshi et al. 2007) suggest to
 109 focus the research towards reactive oxygen species (hereinafter: ROS) and the enzymes involved in their
 110 metabolism. In this perspective, peroxidase activity might be critical because it is involved in the active
 111 oxygen consumption during cell wall anabolisms. Furthermore, metabolomic indicators such as
 112 chlorophylls, carotenoids, tocopherol, ascorbate and glutathione (Tausz et.al. 2001, Tausz et.al. 2007) and
 113 specifically phenol content could represent a supplementary class of valuable biomarkers since their level
 114 and composition is affected by abiotic stress (Jansen et.al. 2014, Ramakrishna and Ravishankar. 2011)
 115 The European beech (*Fagus sylvatica* L.) is one of the most important broadleaved deciduous trees in
 116 central Europe, covering about 12 million ha, and providing a number of ecosystem services. It is widely
 117 distributed from Scandinavia to Southern Italy and from Spain and Southwestern France to Ukraine
 118 (Teissier du Cros et.al. 1981). Distribution of beech trees is likely to change due to modifications in
 119 climate and water availability (Stojanović et.al. 2013) The Scots pine (*Pinus sylvestris* L.) is one of the
 120 most important and widely distributed coniferous species (Bennett. 1984) Vidakovic. 1991).Changes in
 121 quantity and quality of beech and pine wood could have a substantial ecological and economic impacts.
 122 Consequently, the identification of both qualitative and quantitative stress-related biomarkers suitable for
 123 validating different stress models adapted to these species is of outmost importance for forest industry and
 124 farming. Apart from industrial importance, these trees are highly abundant key-species in temperate
 125 central (beech) and boreal European forest ecosystems and therefore represent crucial indicators for better
 126 assessing the overall forest health and ecosystem functioning.
 127 The aim of this study was to identify appropriate biochemical markers able to report the “heat stress”
 128 condition within beech and pine seedlings. This was achieved by comparing several biochemical
 129 parameters in the tissues of seedlings grown at conditions that should reproduce the microclimate of both
 130 natural environment and heat stresses. The biomarkers we looked for were characterized by reliability and

simplicity in terms of manipulation, output, and data analysis since they were selected for clear discrimination between stress classes rather than for their role in plant (stress) physiology.

2. Material and methods

2.1. Plant material

Pine (provenance Velika Gmajna 4.0253; 240-440 m a.s.l., 46°17' N 14°22' E) and beech (provenance Osankarica 2.0119; 1240 m a.s.l., 46°27' N, 15°23' E) were planted on 20.11.2009 and grew outdoors for one year (tree nursery Omorika d.o.o., Muta, N Slovenia) before being transferred to the Forestry Institute in Ljubljana, central Slovenia. Initially, 17 seedlings were selected for each different condition (85 in total). Pines were planted in pots, and beech seedlings were planted as one seedling per rhizotron. Rhizotrons (external size 30x50x3 cm) were packed in boxes and protected from the light. The bottom third of the rhizotrons was filled with sand to allow for water draining, while the upper two thirds were filled with dystric cambisol originated from sandstone and slate ground rock collected from the upper soil horizon (0 to 30 cm) in a mixed forest in the vicinity of the Slovenian Forestry Institute. The soil was sieved through a 5x5 mm sieve, autoclaved and mixed with one third of vermiculite. No fertilizer was used during the experiment. After transplanting, seedling survival was 98% and 95% in the successive growing season. Soil temperature was monitored at a depth of -20 cm using factory calibrated digital temperature sensors DS18B20 connected to a datalogger developed at the Slovenian Forestry Institute. Air temperature and humidity were logged by USB dataloggers Voltcraft® DL-120 TH (Conrad Electronic UK Ltd, Barking, UK). CO₂ concentrations were monitored daily between 11.00-14.00 during the whole growing season 2011 using infrared gas analyzer Li-840 (Li-Cor Inc., Lincoln, USA). Seedlings were collected on 22.8.2012 experimentally measured growth conditions are summarized in Table 1.

Pine seedlings were grown in three different conditions. Control samples (hereinafter CN, 10 samples used for biochemical analyses) were grown outdoors sheltered from direct wind, whereas the

experimental conditions presented either growth in a climatized room (IMP Klima, Godovič, Slovenia) at air temperature of 16-20°C during the day (hereinafter CR, samples used for biochemical analyses) or kept in the greenhouse where mean temperature was 12.3°C in December and Januar and fluctuated between 40 and 47°C in summer months (heat-stress, hereinafter HS, 8 samples used for biochemical analyses) (Table 1).

Beech samples were grown under four different temperature conditions, three were the same as for the pine samples (in all the cases, 7 samples were used for biochemical analyses) while the fourth group was grown in a climatized room (IMP Klima, Godovič, Slovenia) at 16°C, with roots additionally cooled to approximately 12°C (6 samples, hereinafter CR, all 6 samples used for biochemical analyses) by means of a customized refrigerator. The treatment with additional cooling was used to mimic the natural temperature gradient from aboveground to belowground. In the treatment without cooling of the root system, the temperature of the root was practically equal to the temperature of the air. Samples in climatized room were exposed to artificial light of a full PAR spectrum in the range $90 \pm 5 \mu\text{mol m}^{-2}\text{s}^{-1}$. Seedlings grown in the green house were exposed to the natural light in the range $50 \pm 3 \mu\text{mol m}^{-2}\text{s}^{-1}$, while seedlings grown outdoor were exposed to the natural light in the range $120 \pm 20 \mu\text{mol m}^{-2}\text{s}^{-1}$. The daylight regime in the climatized room was adjusted weekly to the natural daylight regime. Beech seedlings were watered with filtered tap water without additional nutrients via an automatic watering system that kept the soil moisture at 10-15% during the growing season, while pine seedlings were watered by hand daily so that the substrates were kept moist according to the consumption. After two growing seasons, leaves and needles were randomly collected, snap frozen in liquid nitrogen, and stored at -80°C until further analysis. Prior to snap-freezing, weight and surface area of fresh material was determined. In total, 53 samples of leaves/needles were measured..

2.2. Homogenization and extraction of plant material

Working temperature throughout the experiment was 4°C. Homogenization was performed on ice using mortar and pestle. Solvents used for homogenization were precooled overnight at 4°C (Bollag, 1996).

Prior to freezing, each individual leaf/needle was weighed and area/length measured. Individual leaves or needles were homogenized using 1mL of appropriate solvent per 0.1 g of weighed material (Table 2). Frozen material was thawed during homogenization procedure in cooled extraction buffer. For analysis of enzymatic activity and protein content plant material was extracted with extraction buffer (50 mM Tris-HCl buffer, pH 7.4), while 80% methanol in water was used for the analysis of phenolics and free radical scavenging activity. Plant homogenate was mixed at 4°C for 2 hours after which it was centrifuged 10 min at 14,000xg at 4°C. Supernatant was used for the experiments after determination of total protein concentration by using Quant-IT™ protein assay (Life Technologies, USA) according to the manufacturer's instructions.

2.3. Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent (Singleton and Rossi. 1965). Ten microliters of extract were mixed with 75 µL of 10-fold diluted Folin-Ciocalteu reagent and incubated 5 min at 22°C before the addition of 75 µL of sodium bicarbonate (0.72 M) solution. Absorbance was measured at 620 nm using a HTS7000 Bioassay reader (Perkin Elmer, USA) after 90 min at 22°C. Results are expressed as galic acid equivalents per mL of solution. Triplicate measurements were performed for each sample.

2.4. Free Radical Scavenging Activity (FRSA) test

The ability of plant methanol extract to scavenge free radicals was determined using di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) assay. The DPPH assay was performed according to the method developed by Brand-Williams (Brand-Williams et.al. 1995). A solution of 50 µM DPPH in 80% (v/v) methanol was stirred for 40 min. Then, 0.05 mL of standard or sample was mixed with 0.1 mL of DPPH solution and incubated for 30 min in the dark after which the absorbance at 492 nm was detected using a Perkin Elmer HTS 7000 spectrophotometric reader. The concentration of DPPH in the reaction medium was calculated from a calibration curve using galic acid as a reference (concentration of 10 to 100 µg/mL

in an 80% methanol solution, tested under the same conditions). The total free radical scavenging activity was expressed as μg galic acid equivalents per mL of sample. All measurements were carried out in triplicate.

2.5. Measurement of enzyme activities

Peroxidase activity (POX) was detected using o-dianisidine (Sigma-Aldrich, Germany) as a substrate (Pine, Hoffman. 1984). The reaction mixture was prepared by mixing 20 mL of 50 mM phosphate buffer, pH 7.0 with 9.79 mM hydrogen-peroxide and 0.2 mL o-dianisidine solution in methanol 11.1 mM. The reaction was initiated by adding plant extract (50 μL) to the reaction mixture (900 μL) in the measuring cuvette. The mixture was mixed and the change in absorbance at 430 nm was read for 5 min at RT using a Perkin Elmer lambda 35 UV/Vis spectrophotometer (Perkin Elmer, USA). One unit of peroxidase activity was defined as the amount of the enzyme that oxidizes o-dianisidine into 1 μM of bis-(3,3'-dimethoxy-4-amino) azodiphenyl per min at 25°C with the extinction coefficient 30 $\text{mM}^{-1} \text{cm}^{-1}$. Triplicate measurements were performed for each sample.

Superoxide dismutase (SOD) activity was assayed by its ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT, Serva, Germany) to blue formazan (Winterbourn et.al. 1975). The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 0.66 mM EDTA, 10 mM L-methionine, 33 μM NBT, and 3.3 μM riboflavin. The reaction was initiated by adding plant extract (50 μL) to the reaction mixture (200 μL). After mixing, samples were illuminated with sunlight for 10 min and absorbance at 492 nm was recorded using HTS7000 Bioassay reader (Perkin Elemer, USA). Blank was prepared by mixing extraction buffer with reaction mixture and kept in the dark while positive control was prepared in the identical manner and exposed to sun light same as the samples. One unit was defined as the amount of protein causing a 50% inhibition of NBT photoreduction. Triplicate measurements were performed for each sample.

Glutathione peroxidase (GPX) activity was assessed by measuring the H_2O_2 -dependent oxidation of glutathione GSH into GSSG (Wendel. 1980). GSSG content was then determined in a coupled reaction in which glutathione reductase reduces the substrate into GSH oxidizing NADPH into NADP. The reaction mixture contained 48 mM sodium phosphate, pH 7.8, 0.38 mM EDTA, 0.12 mM NADPH, 3.2 U of glutathione reductase, 1 mM GSH, 0.02 mM DL -dithiotritol, and 2.28 mM H_2O_2 . The rate of NADPH oxidation measured at 340 nm for 3 min was recorded using a HTS7000 Bioassay reader (Perkin Elemer, USA). One unit is defined as the amount of protein able to catalyze the oxidation of 1 μM of GSH per min at pH 7.0 and RT. Triplicate measurements were performed for each sample.

2.6. Native PAGE and SDS PAGE

For in-gel analysis of enzymatic activity, aqueous plant extracts were resolved under non-reducing conditions in a discontinuous buffer system using a vertical electrophoresis slab system (Hoefer, Holliston, USA) with a 4 % (w/v) stacking and a 10 % (w/v) resolving gel (Table 2). Each gel lane was loaded with 12.5 μg of total protein. POX activity was identified according to a modified Quesada protocol (Quesadaet.al. 1990). After electrophoresis, the gel was washed twice with 50 mM acetate, pH 6.0, after which it was incubated 1 h in 50 mM acetate, pH 6.0, 28 mM o-dianisidine, and 36.4 mM H_2O_2 . SOD activity after native PAGE was detected as previously described (Beauchamp and Fridovich. 1971)

For the analysis of protein profiles, plant samples were resolved using reducing SDS-PAGE and the same gel conditions and protein amounts described above. Gels were stained with Coomassie Blue (Serva, Heidelberg, Germany).

2.7. Statistical analyses

Statistical analysis was performed using GraphPad Prism v5.03 for Windows (San Diego, California, USA). A significance level of $p \leq 0.05$ was used for analysis of variance, implemented using the Kruskal-Wallis test followed by the Tukey's post-hoc test ($p \leq 0.05$). Correlation between different parameters was performed at significance level of $p \leq 0.05$. Comparable percentage was calculated by the formula 3

(%)= (100* sample/ control)-100 with positive values in the case of increase and negative values in the case of decrease in comparison to the control. Unsupervised hierarchical clustering and heatmap generation was accomplished using XLSTAT, with the Manhattan method and Pearson correlation for the distance measure. For heatmap generation, measured values were standardized across the two cohorts by conversion to Z-scores (peak height-mean/standard deviation).

3. Results

3.1. Morphological and biomarker changes in beech samples during temperature stress

Each individual leaf/needle (fresh plant material) was weighed and its area/length measured prior to freezing (Fig. 1, Fig 5.). Significant differences in fresh weight (FW) were observed only in the cases of beech CR . Specifically, beech CR seedlings produced heavier leaves (comparable percentage change +93,05%, Fig 1A) No significant deviation from the control sample group was observed when leaf area was measured (Fig. 1B).

A significant increase in phenolic content relatively to the control was observed for both CR and HS samples (CR +43.81%, HS +37.33%), while CS samples did not show any significant change (Fig. 1C, Fig. 5). A clear difference is observed in the beech plant extract ability to scavenge free radicals. In CS plants it dropped significantly (-6.70%) while the free radical scavenging activity largely increased in CR (+101.65) and HS (+97.93%) samples in relation to control samples (Fig 1D, Fig. 5).

The POX specific activity was clearly affected by artificial growth conditions (Fig. 2A, Fig. 5A). A significant decrease in POX specific activity was observed when comparing the samples grown outside (CN) with samples grown inside the climatized room both with and without root cooling (CS and CR, by -51.71% and -59.62%, respectively). The statistical significance of the POX activity variation further increased in samples treated to reproduce a heating stress (HS, by -70.49%). SOD specific activity decreased significantly in the samples subjected to all of the applied conditions (Fig. 2B, Fig. 5A) when

they were compared to control plants (CS -46.16%, CN -62.47%, HS -62.96%) . GPX activity increased only in beech CS samples (+97.52%) (Fig. 2C, Fig. 5A)..

Since plants possess several POX isoforms, we attempted to separate them using a native gel system to possibly identify which of the enzyme isoforms might be an appropriate biomarker specific for the different growth temperature. A clear difference in the POX expression pattern was observed for beech samples (Fig. 2D, POX). In The majority of POX activity is performed by isoforms with apparent high molecular weight, whereas the contribution of low molecular weight isoforms seems to be negligible. In the CS samples, a slight increase in the activity of low molecular weight isoforms was observed with no apparent decrease in activity of the high molecular weight isoforms. In contrast, the CR seedlings showed both the loss of some among the high molecular weight isoforms and a relevant enhancement of the low molecular weight isoform activity. A qualitatively different POX isoform profile was identified also in the samples extracted from seedlings subjected to heat stress (HS).

Native PAGE was used also to separate SOD isoforms (Fig. 2D, SOD). However, for all of the samples no exploitable difference in isoform profile was observed and the measured differences in specific activity seems rather to be attributed to the different level of expression of the same SOD isoform.

Such information could be integrated by the analysis of the sample total protein profile determined by SDS PAGE that indicated distinctive differences in all of the analyzed beech samples (Fig. 2D, SDS).

3.2 Morphological and biomarker changes in pine samples during temperature stress

Changes in pine needle size was observed only in case of CS plants (change of -59,38%) (Figs. 3A and 5B) while HS samples were unaffected. Similar results were observed for needle weight. CS plants showed decreased needle weight (-59,38%, Fig 3B and 5B) while HS again showed no significant change. In the case of phenolic content, no statistically significant difference between the samples and the control was observed in pine samples (Fig. 3B, Fig. 5B). A highly significant increase in free radical scavenging activity was noted only for the CS seedlings (+65.66%) (Fig 3B, Fig. 5B).

POX activity in pine seedlings was unaffected by heat stress response (Fig. 4A, Fig. 5B), whereas a dramatic decrease was recorded for seedlings which grew in strictly controlled conditions (change of -96.67%) throughout their growing season. The most significant decrease in SOD specific activity was observed in seedlings subjected to heat stress (Figs 4B and 5B, -54,60%). Only heat stress conditions (HS) induced a decrease in GPX activity (Fig 4C-29.86%) while such enzymatic activity remained unaffected in seedlings that underwent growth conditions in which air temperature was controlled (Figs 4C and 5B; CS).

Native PAGE was used for evaluation of POX specific activity of pine seedling extract (Fig 4D, POX). There is an observable difference among the tested samples that seems to be due to the relative expression/activity level of the isoforms rather than to the appearance/disappearance of specific isoforms. Nevertheless, the overall patterns appear very specific for each of the growth conditions. Same as in the case of beech samples, analysis of total protein profile via SDS-PAGE shows a distinct profile (Fig 4D, SDS) for each of the analyzed samples.

4. Discussion

In this study we evaluated the possibility of using simple biochemical techniques to recover reliable data able to differentiate control seedling samples from plants subjected to different temperature stresses and applied this analysis to two industrially important and highly abundant tree species, the European beech and the Scots pine. In this perspective, the choice of the biomarkers can be totally arbitrary and their physiological relevance could be also minimal. What is really crucial is their capacity to act as specific indicators of a stress condition by clearly discriminating between stressed and control samples. Furthermore, biomarkers should be robust in the sense that their recovery, processing, and analysis should be simple and allow for large-scale data comparison among samples collected under very variable field conditions. The evaluation of POX activity and its isoforms was of logical choice and we thought to integrate it with the analysis of other molecules involved in ROS scavenging and cell-wall metabolism.

The statistical analysis indicates that both enzyme biomarkers such as peroxidases, superoxide dismutases, and glutathione peroxidases and phenolic compounds were able to discriminate between stressed and control seedlings. Dendrogram and accompanying heatmap further depict the relative incidence of biomarkers in stressed and control seedlings (Fig. 5). There is a very high correlation between SOD and POX specific activity in beech samples ($r^2=0.9889$, $p= 0.0055$). A strong positive correlation in beech is also observed for phenolic content and leaf weight ($r^2= 0.9758$, $p= 0.0121$) as well as phenolic content and FRSA ($r^2=0.9157$, $p= 0.0421$), and FRSA and leaf weight ($r^2=0.9430$, $p= 0.0284$). This is further corroborated by clear clustering of these parameters in the heatmap (Fig. 5A). In pine samples strong positive correlation is observed between GPX and SOD activity ($r^2=0.9988$, $p= 0.0152$), GPX and phenolic concentration ($r^2=0.9950$, $p= 0.0316$), SOD and phenolic concentration ($r^2=0.9886$, $p= 0.0163$), and between leaf size and weight ($r^2=0.9923$, $p= 0.0393$). This is also clearly visible in dendrogram and accompanying heatmap (Fig. 5B).

Plant responses to abiotic stresses comprises morphological, physiological, and biochemical changes that contribute to counteract the damages and facilitate the recovery of the impaired systems (Potters et.al. 2007, Stojnic S.et.al. 2015.). Recent studies revealed that plants are able to develop distinct morphological responses (stress-induced morphogenesis responses – SIMRs) when challenged with abiotic stresses (Potters et.al. 2007, Potters et.al. 2009). Different stressors were found to induce similar morphogenic responses such as inhibition of cell elongation, localized stimulation of cell division, and modification of cell differentiation (Potters et.al.2007). In other cases, increased temperature and drought did not affect significantly plant biomass and did not cause variations of the weight and surface of the leaves/needles recovered from the treated seedlings in Douglas-fir seedlings (Jansen et. al. 2014). On the other hand, soil temperature influences beech root length, root tip density, biomass of shoots and roots (Štraus et.al. 2014), while stomal density (Stojnic. et. al. 2015.) seems to be strongly influenced (Štraus et.al. 2014) by water availability. In contrast, the enzymes involved in the response to oxidative stress as well as the phenolic compounds (Jansen et al. 2014) seem to be more suitable to be exploited as biomarkers to monitor temperature-induced physiological modifications. Both controlled air temperature conditions and over-

heating resulted in the very strong inhibition of POX and SOD total activities in beech samples. At least in the case of POX, also the isoform expression and their activity profile could be easily used to gain information concerning the level of stress within seedlings. The other tested enzymatic activities and compound concentrations seem to be less suitable stress sensors in the sense that significant variations have been measured only in particular cases, such as the increase of GPX activity in CS and the higher phenol content and free radical scavenging activity specific of CR and HS beech plants. The measured variations are confirmed by previous reports which indicated that plants most probably accumulate secondary metabolite and enhance antioxidant capacity during acclimation (Cansev et.al. 2012) and in response to higher temperatures (Rivero et.al. 2001).

The identification of reliable biomarkers for pine stress was less straightforward. Nevertheless, POX activity strongly declined in the presence of CS conditions, whereas SOD – and to a smaller extent GPX - activity dropped in the presence of heat stress. Native gels stained for POX activity were useful to discriminate between control and stressed samples since the intensity of the different isoforms was strongly affected.

Total phenolic content was not significantly altered by growth conditions while FRSA test could be considered as a biomarker in the case of stress induced by cold growth conditions.

In this work we aimed at identifying robust and reliable biomarkers to monitor stress conditions induced by environmental factors. The elucidation of the physiological modifications that happen in beech and pine under the stress conditions induced in our experiments is beyond the aim of the present contribution.

For instance, enzymes such as POX play both a scavenging activity and an anabolic role in the lignin biosynthesis using hydrogen peroxide as a substrate (De Marco and Roubelakis-Angelakis. 1996) and consequently the separate analysis of the activity and cellular localization of the different isoforms would be necessary to understand their biological meaning . Used as a biomarker, POX enables to obtain very clear and reproducible quantitative (specific activity) and qualitative (isoform profile) data to discriminate samples by means of simple technical analyses. Quantitative measurements are useful to obtain data for modeling whereas the isoform profile separated by native gels can be very informative to identify

biomarkers specific for different stress factors . Furthermore, our results indicate that coupling “POX fingerprinting” with data relative to one or few other parameters, such as SOD activity, phenolic content and FRSA for beech, and GPX activity and phenolic content in pine, could define particular combinations of stress conditions. The present report might act as a sort of proof-of-principle for the future accomplishment of larger sets of biochemical biomarkers for different species of forest tree species. Assessing the health of individual plant specimens on molecular/metabolic level could then give overall indication of environmental conditions in a forest and allow for the improvement of the methods used for forest health monitoring and management (Trudić et.al. 2012). Given the test simplicity, it would be worthy to apply POX analysis to other relevant stress conditions for forest plants such as the presence of pollutants in the air or in the soil.

5. Conclusions

The collected results indicate the feasibility of identifying biochemical biomarkers to use for evaluating the effect of temperature modification on the seedlings of forest tree physiology. Identified biochemical biomarkers could be integrated with genetic information (Fang et.al. 2014, Liu et.al. 2014) to provide useful data needed for assessing environmental stress and for modeling the ecological impacts of global climate change on European beech and Scots pine across their distribution areas. Furthermore, coupling the variation of peroxidase isoform profile with other enzymatic parameters could represent a simple, reproducible, and possibly more universal assessment method for monitoring the tolerance level to temperature stress (in other forest plant seedlings. This approach developed for seedlings might represent also a promising platform for the evaluation of heat stress in adult forest plants in combination with orthogonal techniques such as genomic and proteomic analyses.

These tools could also improve selection and breeding practices since they simplify the profiling of clones to identify those that are more tolerant/resistant to abiotic stress.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. This research did not involve any Human participants and/or Animals. All the authors have made a significant contribution to this manuscript, have seen and approved the final manuscript, and have agreed to its submission to the Canadian Journal of Forest Research.

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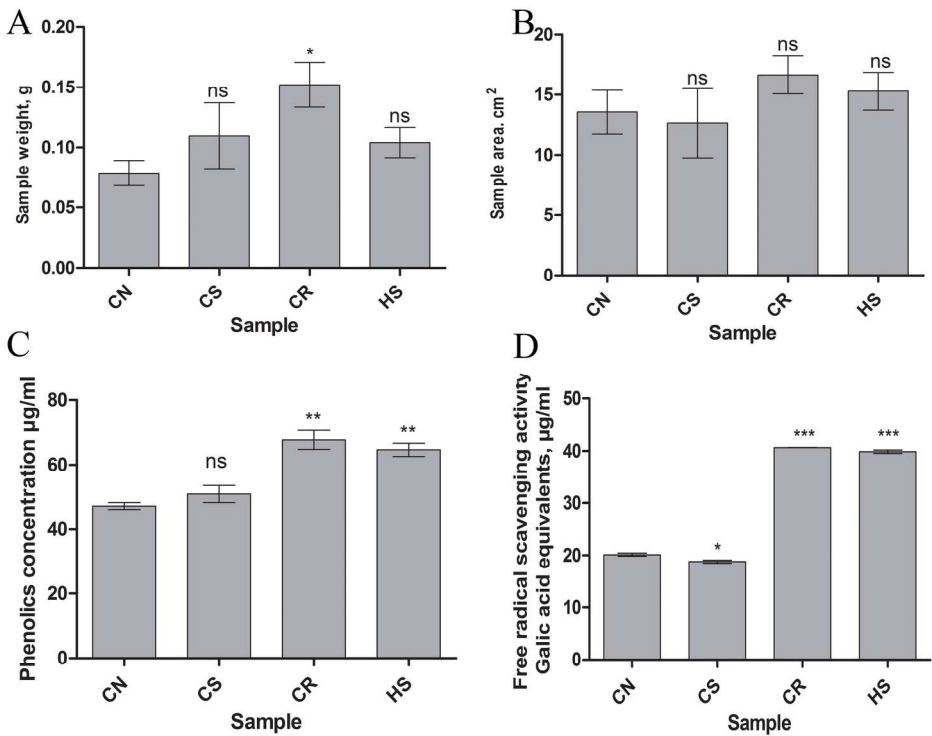


Figure 1. Comparison of morphological characteristics and determination of total phenolic content and free radical scavenging activity of plant methanol extracts for beech seedlings

Plant fresh weight is expressed in grams (A); Plant surface is expressed in cm² (B). Total phenolic concentration in plant methanol extracts is expressed in mg/ml (C); Free radical scavenging activity in plant methanol extract expressed as galic acid equivalents in mg/ml (D). The errors bars indicate standard deviations for triplicate measurements

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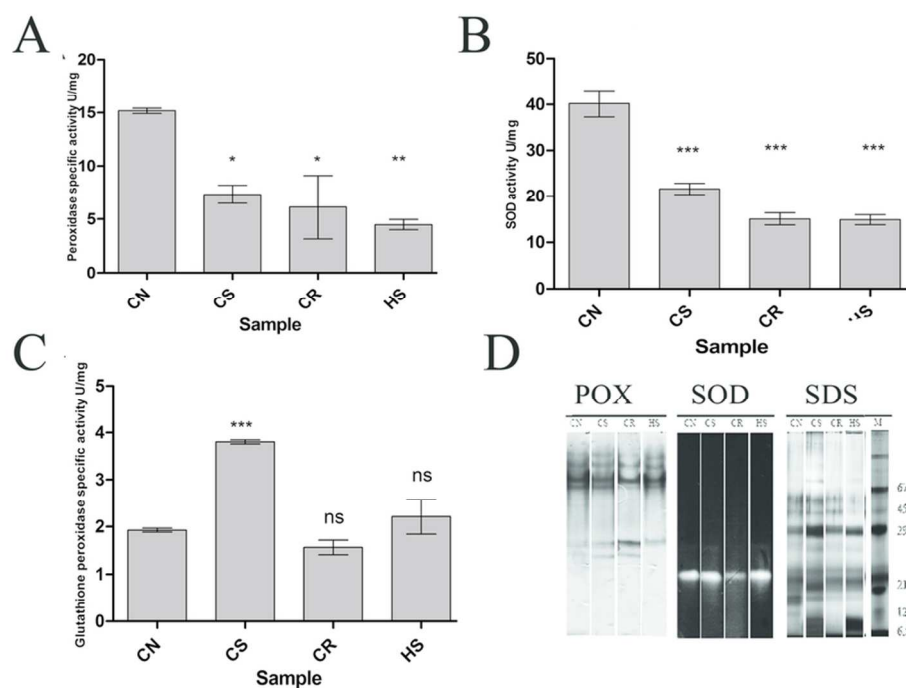


Figure 2. Specific activity of biomarker enzymes in soluble protein extract and electrophoretic separation of biomarker isoforms and total protein content in beech seedlings. Specific enzymatic activities of peroxidase (A), superoxide dismutase (B), glutathione peroxidase (C) are represented in the graph. The errors bars indicate standard deviations for triplicate measurements. Detection of peroxidase (D; POX) and superoxide dismutase (D; SOD) by native PAGE zymography, SDS PAGE (D; SDS) of total soluble extract.

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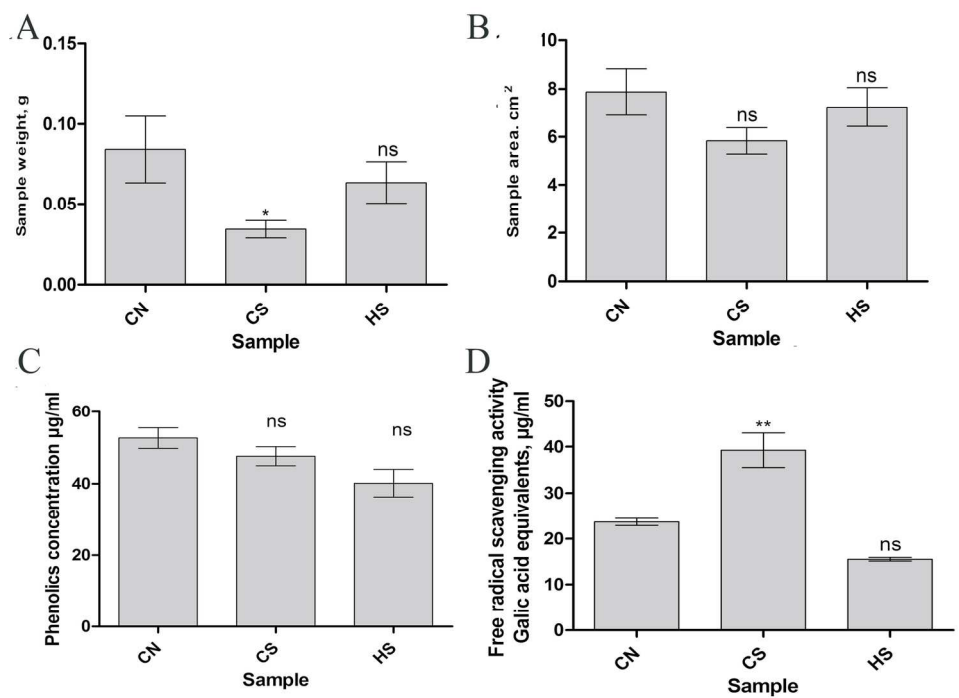


Figure 3. Comparison of morphological characteristics and determination of total phenolic content and free radical scavenging activity of plant methanol extracts for pine seedlings
Plant fresh weight is expressed in grams (A); Plant surface is expressed in cm² (B). Total phenolic concentration in plant methanol extracts is expressed in mg/ml (C); Free radical scavenging activity in plant methanol extract expressed as galic acid equivalents in mg/ml (D). The errors bars indicate standard deviations for triplicate measurements

165x115mm (300 x 300 DPI)

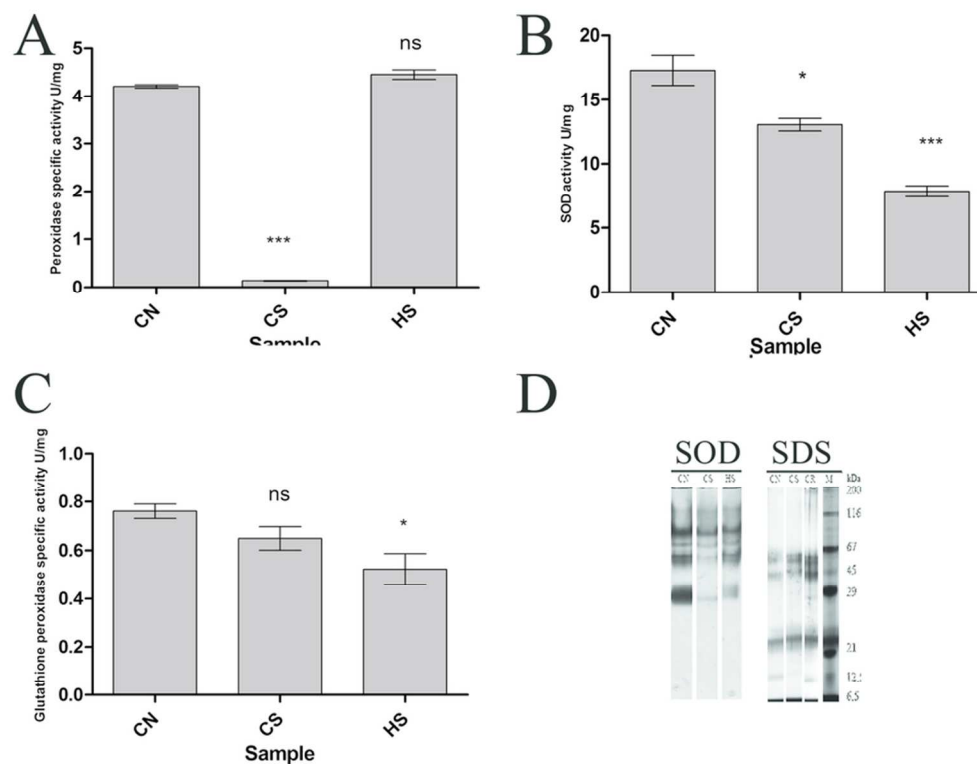


Figure 4. Specific activity of biomarker enzymes in soluble protein extract and electrophoretic separation of biomarker isoforms and total protein content in pine seedlings
Specific enzymatic activities of peroxidase (A), superoxide dismutase (B), glutathione peroxidase (C) are represented in the graph. The errors bars indicate standard deviations for triplicate measurements.
Detection of peroxidase (D; POX) and superoxide dismutase (D; SOD) by native PAGE zymography, SDS PAGE (D; SDS) of total soluble extract.

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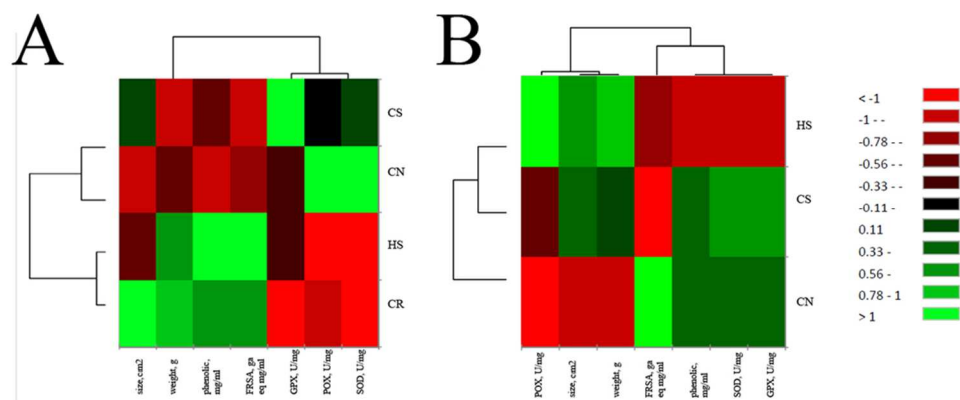


Figure 5. Heat map showing unsupervised hierarchical clustering of different biomarker values according to the seedling growth conditions

Measured biomarkers for beech (A) and pine (B) seedlings are arranged in columns while growth conditions are in rows. Shades of red represent elevation of a metabolite while shades of green represent decrease of a biomarker value, relative to the median levels (see color scale). In the dendrograms, the clustering clearly differentiates the stressed and control samples. Plant fresh weight is expressed in grams (weight, g); Plant surface is expressed in cm² (size, cm²). Total phenolic concentration in plant methanol extracts is expressed in mg/ml (phenolics, mg/ml); Free radical scavenging activity in plant methanol extract expressed as galic acid equivalents in mg/ml (FRSA mg/ml). Specific enzymatic activities of peroxidase (POX, U/mg), superoxide dismutase (SOD, U/mg), glutathione peroxidase (GPX, U/mg) are represented in U/mg of total protein.

93x38mm (300 x 300 DPI)

Table 1: Seedling growth experimental conditions

CN: Samples grown outdoor, CS: Samples grown at 16°C, CR: Samples grown at 16°C with roots cooled to 12°C, , HS: Samples grown in green-house

Means are based on values recorded daily and every half an hour

		Air temperature (°C)			Air RH (%)			CO ₂ concentration (ppm)			Soil temperature at -20 cm depth (°C)		
		Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.
Climatized room	CS	16.0 ± 2.3	13.4	30.1	77.3 ± 5.6	0.5	90.9	671 ± 214	445	1548	15.1 ± 2.5	10.4	25.8
	CR										12.0 ± 2.9	9.1	25.9
Greenhouse	HS	20.1 ± 7.4	10.9	47.1	56.1 ± 14.0	10.3	86.1	532 ± 80	415	792	21.5 ± 5.0	8.6	31.6
Outdoor	CN	10.2 ± 9.0	-12.0	29.6	79.4 ± 15.4	17.8	99.7	413 ± 25	364	482	15.2 ± 6.8	-0.2	26.6

- 15 Table 2: Experimental set-up
- 16 POX: peroxidase, SOD: superoxide dismutase, GPX: glutathione peroxidase, FRSA: free radical scavenging activity

Tree Species	European beech (<i>Fagus sylvatica</i> L.)		Scots pine (<i>Pinus sylvestris</i> L.)	
Morphological features	Leaf size; Leaf weight			
Extraction procedure	50 mM Tris-HCl buffer, pH 7.4	80% methanol	50 mM Tris-HCl buffer, pH 7.4	80% methanol
Biomarker assessment	Enzyme specific activity (U/mg): POX, SOD, GPX	Phenolic concentration (µg/mL)	Enzyme specific activity (U/mg): POX, SOD, GPX	Phenolic concentration (µg/mL)
	Enzyme isoform distribution (Native PAGE): POX, SOD		Enzyme isoform distribution (Native PAGE): POX, SOD	
	Total soluble protein profile (SDS-PAGE)	DPPH RSA test	Total soluble protein profile (SDS-PAGE)	FRSA test